

Iron-sulfur clusters as biosensors of oxidants and iron

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Iron-sulfur clusters are prosthetic groups commonly found in proteins that participate in oxidation-reduction reactions and catalysis. Here, we focus on two proteins that contain iron-sulfur clusters, the fumarate nitrate reduction (FNR) protein of *Escherichia coli* and mammalian iron-responsive-element-binding protein 1 (IRP1), both of which function as direct sensors of oxygen and iron levels. Assembly and disassembly of iron-sulfur clusters is the key to sensing in these proteins and we speculate that iron-sulfur clusters might be found in other regulatory proteins that sense levels of iron and/or oxygen.

IRON-SULFUR PROTEINS are defined as proteins in which the iron is at least partially coordinated by sulfur. In most instances, the iron is either bound to sulfur from cysteine residues in the peptide backbone or to inorganic sulfurs in a prosthetic group known as an iron-sulfur cluster. Iron-sulfur proteins are found in a wide range of organisms from bacteria to man. In many of these proteins, the chief role of the cluster is to facilitate electron transfer, while in others, the clusters contribute to catalytic function, or to the maintenance of structural integrity. Iron-sulfur proteins were not recognized as distinct from other non-heme iron-containing proteins until the 1960s, when analytical techniques, such as electron paramagnetic resonance (EPR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and Mossbauer spectroscopy were applied to these proteins (see Ref. 1 and lit. cit. therein).

The most common types of iron-sulfur clusters are depicted in Fig. 1. The chemical versatility of these prosthetic groups allows them to be used in processes as diverse as nitrogen fixation, photosynthesis and electron transport^{2,3}. Formation of iron-sulfur clusters can proceed spontaneously when sufficient amounts of reduced and soluble iron and sulfur are available⁴, and the prevalence of such conditions early in the history of the earth could account for

the presence of these prosthetic groups in a wide variety of proteins⁵.

Chemical properties

Greater flexibility in catalytic and electron transfer reactions is obtained with iron-sulfur clusters than would be possible with single-metal sites. Some of the characteristics of transition metals retained in the electronic configuration of these clusters include the presence of high-spin iron sites (sites that contain unpaired electrons) and a significant capacity to delocalize electrons, particularly in [4Fe-4S] clusters⁶. These clusters can function as strong reductants; the reduction potentials of clusters are often quite low, and can reach -700 mV. Indeed, iron-sulfur proteins are regarded as the chief villains responsible for cellular generation of superoxide, a by-product of respiration formed by reducing agents that are powerful enough to perform a single-electron reduction of dioxygen. A wide variation in the reduction potentials of clusters, with midpoint reduction potentials varying from -700 mV to +300 mV, is seen². Variations in cluster potentials are attributable in part to characteristics of the individual protein, including the nature of the cluster ligands and the hydrophobicity and charge of residues in the environment of the cluster. For fumarate reductase of *Escherichia coli*, there are three physically distinct iron-sulfur clusters present in a single enzyme, and the reduction potentials vary from -320 mV for the [4Fe-4S]²⁺ cluster, to -70 mV for the [3Fe-4S]⁰⁺ cluster, to between -20 and -79 mV for the [2Fe-2S]²⁺ cluster⁷.

Two stable oxidation states are often spanned by [4Fe-4S] clusters, with net formal calculated oxidation states of either 2+ or 1+ in some instances, or 3+ or 2+ in others. Because these clusters have the capacity to accept or donate single electrons, they are frequently found in enzymes in which single electrons must be supplied or removed to catalyse transformations of substrate, as is the case in a number of mammalian respiratory chain proteins, such as succinate dehydrogenase, NADH dehydrogenase and the cytochrome *bc1* complex². Clusters in which the 1+/2+ oxidation couple is normally spanned cannot be further oxidized to the 3+ oxidation state, as further oxidation results in irreversible decomposition of the cluster⁸.

Instability of iron-sulfur proteins

That iron-sulfur proteins can be unstable in the presence of oxidizing substances was first noted decades ago⁹ and has been described in numerous proteins, for example, nitrogenase and hydrogenase¹⁰. Although the actual mechanisms of cluster disintegration are not known, a [3Fe-4S]¹⁺ intermediate and a polysulfide-containing degradation product are produced in the oxidative degradation of mitochondrial aconitase^{11,12}. Work with synthetic clusters has further affirmed the generalization that iron-sulfur clusters are easily destroyed by oxidation¹³. Although much work has been devoted to elucidation of potential synthetic pathways for iron-sulfur clusters, very little work has focused on pathways of spontaneous degradation.

As many iron-sulfur clusters are unstable to oxidation, it is somewhat surprising that these clusters are commonly found not only in anaerobic organisms, but also in aerobic organisms, where many of them perform key functions. Not all protein-bound iron-sulfur clusters are unstable to oxidation. Those proteins that contain stable iron-sulfur clusters tend to share a common underlying feature: the cluster is bound in a region of the protein that is inaccessible to solvent and oxidants. Recent studies on a bacterial [4Fe-4S] protein, HiPIP from *Chromatium vinosum*, have shown that when the binding pocket of the cluster is modified to become more hydrophilic, the cluster is destabilized, most probably because oxidants dissolved in polar solvents can gain access to the cluster¹⁴.

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In synthetic iron-sulfur clusters, the polarity of surrounding groups is also important to the stability of the cluster¹³. While the mechanism of cluster breakdown is not known, it is reasonable to speculate that loss of electrons, beyond a certain point, results in partial free radical formation at bridging sulfurs and cysteinyl ligands. This is followed by coupling reactions between free radicals and hydrolytic degradation of the oxidized center by solvent.

The disintegration of iron-sulfur clusters in certain settings can lead to loss of activity of the associated protein; for example, fumarase C in *E. coli* is produced under conditions of aerobic growth, but is not an iron-sulfur protein. In this setting, amino acid sidechains of fumarase C provide catalytic function, whereas an anaerobically produced form of the enzyme requires an iron-sulfur cluster for function^{15,16}. In those instances in which an alternative stable form of the protein is not synthesized under conditions of oxidative stress, the loss of function of iron-sulfur proteins can lead to toxic effects in cells. In *E. coli*, target enzymes such as aconitase¹⁷, dihydroxyacid dehydratase¹⁸ and 6-phosphogluconate dehydratase¹⁹ are inactivated by the superoxide anion, and the loss of function of these enzymes could contribute significantly to the toxicity observed under conditions of oxidative stress¹⁵.

Although the instability of clusters might be detrimental in some instances, the purpose of this review is to focus attention on several examples of iron-sulfur proteins in which this potential flaw might be advantageous. When there is a need for sensing of oxygen levels, it appears that in at least one situation, the fumarate nitrate reduction protein (FNR) of *E. coli*, the loss of integrity of the cluster in the presence of oxygen is the key to sensing.

The FNR transcription factor of *E. coli*: an oxygen sensor

In response to lowered oxygen tension, the metabolism of *E. coli* switches from use of oxygen to use of alternative terminal electron acceptors such as fumarate and nitrate, a change that requires the simultaneous transcriptional activation of over 50 genes of anaerobic metabolism. Oxygen levels are sensed by the *E. coli* transcription factor FNR. In the absence of oxygen, FNR binds to promoters of the genes of anaerobic metabolism and activates transcription. For many years it was unclear how this

change in binding activity occurred, as total amounts of FNR were unchanged. The ability of FNR to activate transcription was observed to be impaired by iron deprivation, and the relationship between nutritional iron status and oxygen sensing led to speculation that an iron co-factor was involved in sensing of oxygen levels¹⁹.

Recently there has been substantial progress in determining how the function of FNR is regulated: sensing of oxygen depends on the sensitivity of a [4Fe-4S] iron-sulfur cluster to oxygen. The cluster is destroyed within seconds upon exposure to oxygen and an apoprotein devoid of the cluster is the product of the reaction^{20,21}. Interestingly, the discovery that an iron-sulfur cluster was the key to function did not occur until several mutants of FNR were analysed²². These mutations partially stabilized the iron-sulfur cluster in the presence of oxygen, so that iron and inorganic sulfide could be detected. When strict anaerobic conditions were imposed during purification, it became clear that each monomer binds a [4Fe-4S] cluster and dimerized holoprotein promotes transcription of genes of anaerobic metabolism. In the presence of oxygen, the cluster disintegrates, and the protein no longer specifically binds DNA²⁰. Thus, FNR provides a clear example in which a reactive iron-sulfur cluster is used to sense concentrations of the destabilizing agent, oxygen.

Many other proteins have iron-sulfur clusters that are destabilized by exposure to oxidants. The defense against superoxide anions and nitric oxide in *E. coli* is mediated by Sox R, and although the exact mechanism of sensing has not yet been determined, iron-sulfur clusters appear to be key to the sensing process^{23,24}.

Non-regulatory iron-sulfur proteins are also affected by oxidants. In glutamine phosphoribosylpyrophosphate amidotransferase, the enzyme of *Bacillus subtilis* that catalyses the first committed step of purine biosynthesis, [4Fe-4S] clusters contribute to maintenance of the tertiary structure of the protein and exposure to oxygen results in loss of the iron-sulfur cluster, unfolding of the protein and degradation²⁴.

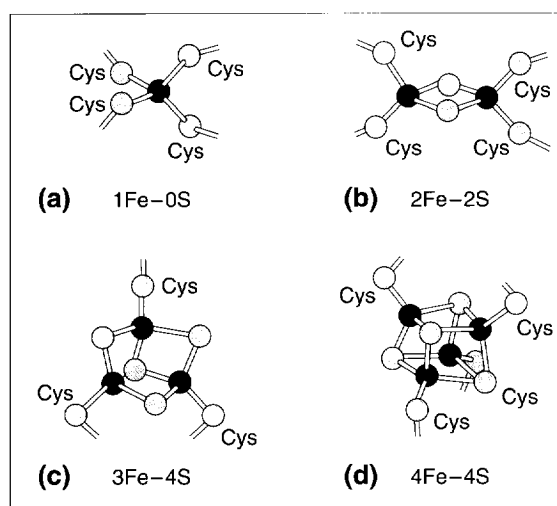


Figure 1

The major forms of iron-sulfur cluster are depicted: (a) the mononuclear iron (red) is ligated by sulfurs of four cysteines (yellow); (b) in the [2Fe-2S] cluster, the two irons are bridged to one another by two inorganic sulfur atoms and ligated to four cysteines as shown; (c) the [3Fe-4S] form is cubane, with a single iron of the cube absent; and (d) the more common [4Fe-4S] form is also cubane.

Other enzymes of *E. coli* containing [4Fe-4S] clusters that are destabilized by reactive oxygen species are fumarase, 6-phosphogluconate and dihydroxyacid dehydratase¹⁵.

IRP1/aconitase senses iron levels

Iron is indispensable to the function of eukaryotic cells, and the uptake and sequestration of iron is regulated by a set of genes that are highly conserved in mammalian cells. Synthesis of the transferrin receptor (TfR), which is responsible for iron uptake, is regulated to respond to metabolic needs. When intracellular iron supplies are adequate, the mRNA for the TfR is rapidly degraded, resulting in a decrease in TfR biosynthesis. When intracellular iron levels are low, mRNA levels and biosynthesis of the TfR increase, while the rate of translation and biosynthesis of ferritin, an iron-sequestration protein, decreases. These regulatory changes are mediated by a post-transcriptional regulatory system that involves binding of iron-regulatory proteins to stem-loop structures in the mRNA known as iron-responsive elements (IREs). Functional IREs are found in the 5' untranslated region (UTR) of the genes that encode ferritin and erythrocyte aminolevulinic acid synthase (eALAS), the rate-limiting step in heme biosynthesis, and in the 3' UTR of the gene encoding TfR. When cellular iron levels are low, iron regulatory proteins bind to IREs, where they

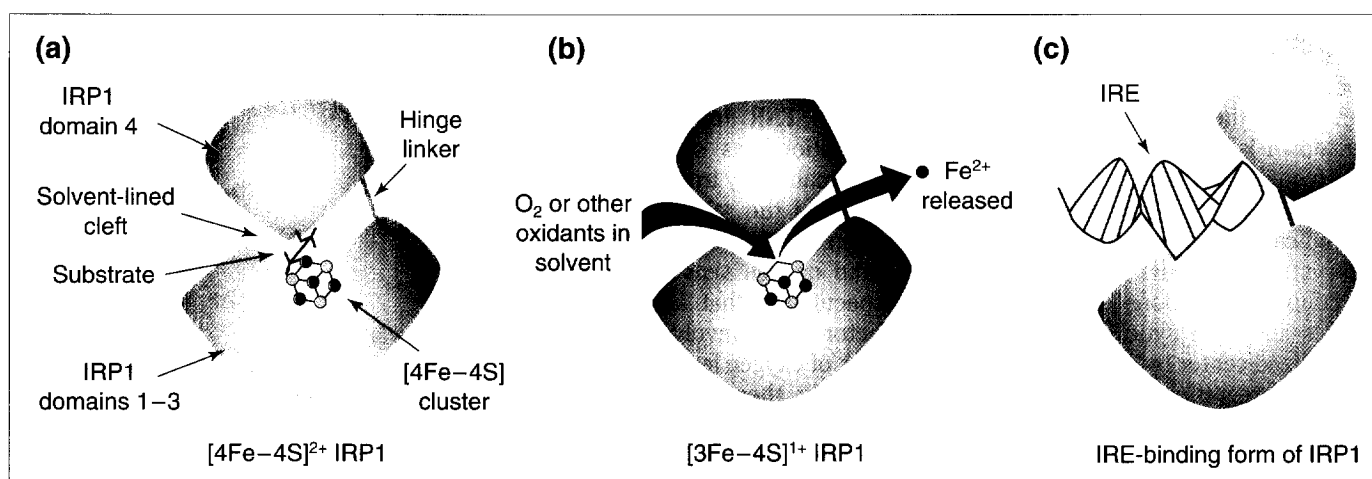


Figure 2

A schematic model of the effect of oxidation on the iron-sulfur cluster of iron-responsive-element (IRE)-binding protein-1 (IRP1), based on studies of the disassembly of the iron-sulfur cluster of mitochondrial aconitase. **(a)** The form with cytosolic aconitase activity contains a solvent exposed $[4\text{Fe}-4\text{S}]^{2+}$ cluster. Oxidation leads to spontaneous release from the cluster of the single Fe^{2+} , which is not bound by a cysteinyl ligand. **(b)** A relatively stable $[3\text{Fe}-4\text{S}]^{1+}$ intermediate is found in aerobically purified mitochondrial aconitase and in oxidized IRP1 (Ref. 35). Further oxidation results in disintegration of the cluster with almost complete loss of iron. Sulfur remains entrapped in polysulfides in the oxidized protein, but is readily released upon reduction^{33,35}. **(c)** The apoprotein is the IRE-binding form and residues in the active site cleft are required for binding (see text).

inhibit translation, (ferritin and eALAS) or mRNA degradation (TfR) (see Refs 26, 27). Tight regulation of iron uptake and distribution is necessary because excess iron can be toxic, particularly because iron species and oxygen can interact to form reactive oxygen species, including superoxide and hydroxyl radicals.

The initial purification of the IRE-binding protein, now called IRP1, has led to insights into the mechanism by which iron levels are sensed²⁶. Human IRP1 was shown to have significant²⁸ (approximately 30%) sequence homology to mitochondrial aconitase, an enzyme that has been crystallized and extensively characterized. Mitochondrial aconitase is known to contain an iron-sulfur cluster, a finding that was initially revealed by Mössbauer spectroscopy²⁹ and was later confirmed biochemically and crystallographically^{30,31}.

A $[4\text{Fe}-4\text{S}]$ cluster like that of mitochondrial aconitase was found in IRP1 (Ref. 32), and analysis of recombinant IRP1 led to the observation that the protein functioned as a cytosolic aconitase in iron-replete cells, whereas it functioned as a high affinity IRE-binding protein in iron-depleted cells (see Fig. 2). These changes in activity occurred without significant changes in amounts of immunologically detectable IRP1 (Refs 33, 34), and use of previously established methods for the *in vitro* assembly or disassembly of the iron-sulfur cluster of mitochondrial aconitase revealed that the two forms of this bifunctional protein could be interconverted

*in vitro*³⁵⁻³⁷. These studies demonstrated that reversible changes in the status of the iron-sulfur cluster accompanied the switch from enzymatic activity to RNA-binding activity in IRP1. The conclusion that the iron-sulfur cluster was key to function was supported by studies showing that mutations of cysteine residues involved in cluster ligation produce apoprotein that binds RNA constitutively, regardless of the iron status of the cell^{38,39}. A variety of studies, including UV crosslinking and sequencing of peptides crosslinked to IRE, have shown that the IRE-binding site overlaps with the aconitase active site cleft⁴⁰⁻⁴². Site-directed mutagenesis studies have shown that arginine residues in the enzymatic active site are also indispensable for high-affinity RNA binding³⁸, thus explaining why the enzymatic activity and RNA-binding activities are mutually exclusive properties of the bifunctional IRP1.

Does IRP1 integrate the sensing of oxidants and iron?

In the example of FNR, cluster disassembly results from exposure to oxygen, and although iron sufficiency is required in sensing, the level of oxygen in the cell is the primary stimulus to which the regulatory response is oriented. In the case of IRP1, however, the major regulatory response is in the control of synthesis of proteins of iron metabolism. To understand how an iron-sulfur cluster can function in sensing of iron levels, we need to understand the

impact of oxidants on the iron-sulfur cluster of IRP1 and understand how changes in iron availability and exposure to oxidants influence the status of the cluster.

The iron-sulfur cluster of IRP1 is positioned in a solvent-filled cleft where it could be subject to destabilization by oxidants and solvent. Treatment of cells with nitric oxide or agents that produce nitric oxide results in activation of IRE-binding activity^{43,44}. Furthermore, treatment of cells with hydrogen peroxide leads to activation of RNA-binding activity and loss of cytosolic aconitase activity, as would be expected if oxidative stress were leading to oxidative disassembly of the iron-sulfur cluster⁴⁵⁻⁴⁷.

An elegant series of experiments on the assembly of synthetic iron-sulfur clusters has resulted in characterization of pathways for the spontaneous orderly assembly of several types of clusters, including $[4\text{Fe}-4\text{S}]$ clusters⁴. However, as is the case with many processes that are spontaneous under optimal conditions, there is evidence that assembly of iron-sulfur clusters is enzymatically catalyzed in bacteria⁴⁸ and that mammalian homologs to the genes encoding these proteins exist (T. Land, R. D. Klausner and T. A. Rouault, unpublished). Therefore, the state of the cluster in cytosolic aconitase might reflect a balance between cluster disassembly, which is likely to occur under conditions of normal aerobic growth, and cluster reassembly, which would require sufficient iron and sulfur, along

with assembly enzymes, as is illustrated in Fig. 3. The relative efficiencies and sensitivities of the various components would determine whether the sensing is primarily for oxygen or for iron, although clearly the sensing of each reactant would require the availability of the other. In this fashion, IRP1 and perhaps other proteins, such as FNR, simultaneously sense levels of oxidants and iron. Although levels of iron might have little impact on the process of cluster disassembly, iron levels determine whether the cluster can be re-assembled and thereby control the transition from apoprotein to holoprotein. The holoprotein can function as a sensor of oxidants, while the apoprotein can function as a sensor of iron levels, as is indicated in Fig. 3. The mechanisms of assembly and disassembly of iron-sulfur clusters are the key to understanding how iron-sulfur proteins can serve as sensors of both oxidants and iron.

Concluding remarks

Iron-sulfur proteins can function as direct sensors of ambient levels of both iron and oxygen in the environment. The chemical reactivity of iron-sulfur clusters may explain why the regulatory responses to environmental fluctuations in these substances are integrated and interdependent. There is much to be learned about the processes of cluster synthesis and degradation, as both processes appear to be important in dynamic sensing. It is likely that iron-sulfur clusters will be discovered in other regulatory proteins that sense levels of iron and oxygen, and it is also clear that oxygen exposure is a critical variable that must be controlled when the key to such regulation is being sought.

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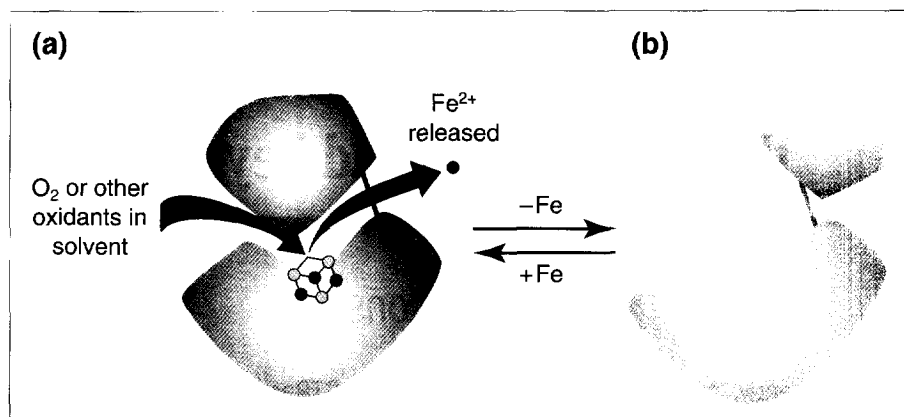


Figure 3

Sensing of iron and oxidants is interdependent. A balance between apoprotein and holoprotein can be represented as the sum of forces contributing to disassembly and those contributing to assembly, including components and enzymes. Depicted are (a) the holoprotein and (b) the apoprotein forms of iron-responsive-element (IRE)-binding protein-1 (IRP1), which function as cytosolic aconitase or IRE-binding protein, respectively. The primary role of a given protein might be to function as either an iron sensor or an oxygen sensor, although the balance achieved will depend on levels of both iron and oxidants. Reassembly of a dynamic iron-sulfur cluster permits sensing of iron levels, while disassembly of an oxidation-sensitive iron-sulfur cluster could permit sensing of levels of oxidants.

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